ENHANCED PRODUCTION OF INDUSTRIAL ENZYMES IN MUCOROMYCOTINA FUNGI DURING SOLID-STATE FERMENTATION OF AGRICULTURAL WASTES/BY-PRODUCTS

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Cellulolytic, lipolytic and proteolytic enzyme production of zygomycetes *Mucor corticolus, Rhizomucor miehei, Gilbertella persicaria* and *Rhizopus niveus* were investigated using agro-industrial wastes as substrates. Solid-state cultures were carried out on untreated corn residues (stalk and leaf) as single substrate (SSF1) or corn residues and wheat bran in mixed fermentation (SSF2). Rapid production of endoglucanase (CMCase) was observed with maximal activity reaching after about 48-h fermentation, while cellobiohydrolase (CBH) and β-glucosidase enzymes generally had their peak after 72-h incubation. Highest filter paper degrading (FPase), CMCase, CBH and β-glucosidase activities obtained were (U g^{-1} dss) 17.3, 74.1, 12.2 and 158.3, for *R. miehei, G. persicaria, M. corticolus* and *Rh. niveus,* respectively. *M. corticolus* proved to be the best lipolytic enzyme producer in SSF1 presenting 447.6 U g^{-1} dss yield, while *R. miehei* showed 517.7 U g^{-1} dss activity in SSF2. *Rh. niveus* exhibited significantly greater protease production than the other strains. Suc-AAPF-*p*NA hydrolyzing activities of this strain were 1.1 and 1.96 U g^{-1} dss in SSF1 and SSF2, respectively. We conclude that the used corn stalk and leaf residues could potentially be applicable as strong inducers for cellulase and lipase production by Mucoromycotina fungi.

Keywords: Zygomycetes – hydrolytic enzymes – corn stalk and leaf – wheat bran – mixed fermentation

INTRODUCTION

Agro-industrial processes produce large quantities of corn stalk and leaf residues as plant-waste materials each year. Solid-state fermentation (SSF) holds huge potential for bioconversion of cellulosic materials and for high-yield enrichment of biomass-degrading enzymes [2, 33]. Therefore, this technology provides an alternative and economical approach to exploit of these renewable and inexpensive resources. Although corn stalk is commonly used for production of biofuels and other chemicals [26], direct bioconversion has poorly been studied up to now. Moreover, in most

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enzymatic digestibility researches, the raw substrate is subjected to chemical, biological or steam explosion pre-treatments [19, 36].

Conversion of cellulosic biomass to ethanol requires enzymatic hydrolysis of this biopolymer to fermentable glucose as a first step. This degradation is carried out by cellulases that are complex mixtures of enzymes with different specificities to hydrolyze the β-1,4-glycosidic linkages of cellulose. There are three major types of cellulolytic activities in this extensively studied multienzyme complex [15]: endoglucanases or CMCases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21). Cellulases useful in industrial processes are mainly extracellular enzymes secreted by different types of fungi [5].

Fungal lipases and proteases also have great importance in microbial degradation of agro-industrial wastes [4, 27]. Lipases (EC 3.1.1.3) catalyze the hydrolysis of triacylglycerols, the major constituents of fats and oils, which can widely be found in plant-waste residues. Outcomes of this hydrolysis are free fatty acids, glycerol and partial acylglycerols, which are easily utilizable energy resources for microorganisms. Microbial proteases are capable to catalyze the total hydrolysis of proteins of plant-waste biomass.

Mucoromycotina is a large group of zygomycetes fungi, which are primarily saprotrophic having well-demonstrated growth characteristics on various low-cost resources. Several zygomycetes are recognized as valuable sources of extracellular hydrolases, some of which are utilized in industrial applications [11]. Crude hydrolase preparations derived from fungal sources may efficiently support biotechnological processes. For instance, addition of crude cellulase cocktail to cellulosic residues makes the saccharification process faster and thus bioethanol production can be cost effective and eco-friendly [26]. While the plant-waste induced secretion of the abovementioned hydrolases has extensively been studied in some filamentous fungi [8, 25, 28], much less is known on zygomycetes in this regard [13, 24, 35]. This study has focused to cellulase, lipase and protease production of selected Mucoromycotina strains using corn stalk and leaf plant-wastes as substrates in SSF. At the same time, the yield-enhancing effect of wheat bran was also explored in mixed fermentation assays.

MATERIALS AND METHODS

Organisms and fermentation materials

Mucor corticolus (SZMC 12031), *Rhizomucor miehei* (SZMC 11005), *Gilbertella persicaria* (SZMC 11086) and *Rhizopus niveus* (SZMC 13625) strains were used. Fungal isolates were deposited in the Szeged Microbiological Collection (SZMC, Szeged, Hungary). For fermentation, equal amount of dry corn stalk and leaf were cut into small pieces. These chopped corn residues were then briefly milled in a commercial mill into particles of 1.5 mm mean diameter. Wheat bran was purchased in a local market, and was added in 1:1 ratio to the corn residues in the case of mixed substrate fermentation experiments.

Solid-state fermentation

SSFs were carried out in 250-mL Erlenmeyer flasks containing 5 g of milled corn stalks and leaves (designated as SSF1) or a mixed substrate (designated as SSF2). Five mL distilled water was added to moisturize the solids, and then the media were autoclaved at 121 °C for 30 min. After cooling, each flask was inoculated with 10^6 sporangiospores and incubated at 25 °C or 37 °C (in the case of *R. miehei*) for 12 days. Crude enzyme extraction was performed on every second day with 50 mL of distilled water by shaking at 4 °C for 3 h. Suspensions were squeezed through a filter cloth, then, filtered with Whatman No. 1 paper and centrifuged at 16,200×*g* for 20 min at 4 °C. Enzyme activities were determined in the clear extracts and were expressed as U g^{-1} of dry substrate (dss).

Cellulolytic enzyme assay

Filter paper degrading activity (FPase) was evaluated using Whatman No. 1 filter paper. Endoglucanase (CMCase) activity was analyzed using carboxymethyl cellulose (CMC; Sigma-Aldrich) as a substrate. The reaction mixtures consisted of 200 µL sodium acetate buffer (0.1 M, pH 5.5), 50 µL crude enzyme extract and 2.5 mg filter paper or 1% CMC. In the case of the FPase activity, mixtures were incubated at 50 °C for 1 h. CMCase activity was studied by incubation at the same temperature for 30 min. Concentration of reducing sugar was determined by the dinitrosalicylic acid (DNS) method [20]. One unit of each activity was defined as the amount of enzyme that released 1 µmol of glucose equivalent from the respective raw substrate per minute under the assay conditions. β-Glucosidase and cellobiohydrolase (CBH) activities were measured using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG; Sigma-Aldrich) and *p*-nitrophenyl-β-D-cellobioside (*p*NPC, Sigma-Aldrich) as substrate, respectively. The reaction mixture contained 800 µL sodium acetate buffer (0.1 M, pH 5.5), 100 µL *p*NPG (7 mM) or *p*NPC (4 mM) and 100 µL crude enzyme extract. Reaction mixtures were incubated for 30 min at 50 °C because β-glucosidases of the used strains had their maximum activity under this condition [30]. The reaction was stopped by adding 2 mL of 0.1 M sodium carbonate, and the released *p*-nitrophenol (*p*NP) was measured spectrophotometrically using a Jenway 6800 Double Beam Spectrophotometer (Jenway, Bibby Scientific) at 405 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of *p*NP per min.

Lipolytic activity assay

Lipolytic activity was determined after Kotogán et al. [17] using *p*-nitrophenyl-palmitate (*p*NPP, Sigma-Aldrich). Incubations were carried out at 25 °C for *G. persicaria, M. corticolus* and *Rh. niveus* and 37 °C for *R. miehei* enzymes. One unit of enzyme activity was defined as mentioned above.

Proteolytic activity assay

Extracellular proteolytic activity of the isolates was studied by the use of chromogenic peptidyl-*p*-nitroanilide substrates. Activity of trypsin-like proteases was measured with N-alpha-benzoyl-L-Arg-*p*-nitroanilide (Bz-R-*p*NA) and N-alpha-benzoyl-Phe-Val-Arg-*p*-nitroanilide (Bz-FVR-*p*NA), while chymotrypsin-like protease activity was determined by N-Succinyl-Phe-*p*-nitroanilide (Suc-F-*p*NA) and N-Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Suc-AAPF-*p*NA). All of these substrates were purchased from Sigma-Aldrich. Reaction mixtures were consisted of 160 µL of distilled water (pH 7.0), 20 µL of substrate (5 mM) and 20 µL of crude enzyme extract. Mixtures were incubated at 25 or 37 °C for 30 min in 96-well microtiter plates, and the reaction was stopped by adding 50 µL of 0.1 M sodium carbonate. The released *p*-nitroaniline (*p*NA) was measured spectrophotometrically (ASYS Jupiter HD, ASYS Hitech) at 410 nm. According to the manufacturer's instruction, the molar absorption coefficient of 8800 M^{-1} cm^{-1} for *p*NA was used to calculate the enzyme activity. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmol of *p*NA per min.

Statistical analysis

All experiments were performed in triplicates. Standard deviations of mean values were calculated using Microsoft Office Excel 2007 function and the Sigma Plot (USA) software was used for statistical significance analyses.

RESULTS

Total cellulolytic activity

In both SSF1 and SSF2, maximal amount of reducing sugar was detected on the second day (Fig. 1). The activity was markedly decreased at the fourth day, which was not observed when corn residue was used as the sole substrate (data not shown). FPase activity of *M. corticolus* had similar rate in both SSF1 and SSF2 during the whole fermentation period. Interestingly, *M. corticolus* had the lowest activity in the wheat bran-supplemented medium among the investigated fungi (6.6 U g^{-1} dss) but it showed outstanding FPase capacity when only corn residues were used (7.2 U g^{-1} dss). The order of the FPase activities in the crude extracts after SSF2 was *R. miehei* > *G. persicaria* > *Rh. niveus* > *M. corticolus*, while it was *M. corticolus* > *Rh. niveus* > *G. persicaria* > *R. miehei* in the SSF1 system.
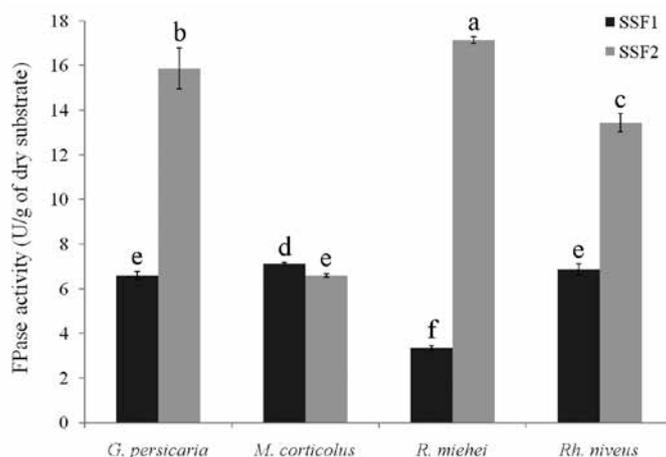
Fig. 1. Filter paper (FPase) activity of zygomycetes strains in corn residue-based (SSF1) and mixed (corn residue and wheat bran) substrate-based (SSF2) solid-state fermentations. Values were measured on the 2nd day of the fermentation. Bars represent SD values of replicates. Different letters over the bars indicate significant differences (P<0.05) in Duncan's multiple range test

Endoglucanase activity

CMCase production of the isolates using SSF1 and SSF2 fermentation systems is shown in Table 1. In SSF2, each isolate showed the highest enzyme yield at the second day of the cultivation. After 48-h incubation, the CMCase activity in the crude extracts was *G. persicaria > Rh. niveus > R. miehei > M. corticolus*. If longer incubation period was applied, enzyme production of *G. persicaria* and *Rh. niveus* isolates remarkably decreased; during a 10-day incubation, their CMCase activities of 74.1 and 59.5 U g^{-1} dss measured on the second day reduced to 52.4 and 46.8 U g^{-1} dss, respectively. In contrasts, after a significant drop by the sixth day (50.5 to 18.6 U g^{-1} dss), a slight increase in the enzyme activity of the *R. miehei* isolate was detected on the tenth day (25.7 U g^{-1} dss). With the exception of *G. persicaria,* usage of SSF1 medium resulted in lower CMCase activities on the first phase of the incubation than those measured on the sixth day. In this medium, the relative CMCase activity in the crude extracts was *G. persicaria > Rh. niveus > M. corticolus > R. miehei*. CMCase activities of *R. miehei* showed big differences in mixed substrate and corn-residue based media, which were not detected in the other tested crude extracts.

Cellobiohydrolase activity

Table 1 shows the time course profiles of CBH activities during the SSFs. It can firstly be observed that the *p*NPC hydrolysis in the crude extract of *M. corticolus* was at least 2–3 times higher than those detected by the other strains in SSF1. Product

Table 1

Time course profiles of cellulase production by four zygomycetous fungi using corn stalks and corn leaves (SSF1) or their mixtures with wheat bran (SSF2) as substrates

	Enzyme yield (U g^{-1} dss)*					
	incubation time (day)					
	2		6		10	
	SSF1	SSF2	SSF1	SSF2	SSF1	SSF2
Endoglucanase (CMCase)						
Gilbertella persicaria	48.8±2.6[a]	74.1±2.7[a]	37.8±1.1[a]	60.5±2.1[a]	32.4±1.6[a]	52.4±2.2[a]
Mucor corticolus	17.2±0.9[c]	32.1±1.8[d]	23.2±0.9[b]	24.7±1.1[c]	19.4±1.2[c]	24.4±1.7[c]
Rhizomucor miehei	4.2±0.1[d]	50.5±1.7[c]	4.3±0.2[c]	18.6±0.9[d]	5.5±0.4[d]	25.7±2.1[c]
Rhizopus niveus	28.8±1.2[b]	59.5±1.9[b]	37.6±1.2[a]	52.9±1.7[b]	21.8±1.3[b]	46.8±2.2[b]
Cellobiohydrolase (CBH)						
Gilbertella persicaria	2.1±0.2[c]	5.7±0.4[a]	1.8±0.2[c]	8.5±0.8[a]	1.7±0.4[b]	6.4±0.4[b]
Mucor corticolus	6.8±0.6[a]	5.5±0.3[a]	9.8±0.6[a]	7.6±0.5[b]	12.2±1.2[a]	8.2±0.4[a]
Rhizomucor miehei	1.2±0.2[d]	1.6±0.2[c]	1.1±0.2[d]	3.7±0.4[d]	1.1±0.2[c]	2.5±0.3[d]
Rhizopus niveus	2.8±0.4[b]	3.6±0.3[b]	2.7±0.3[b]	5.8±0.6[c]	1.6±0.3[bc]	4.7±0.5[c]
β-Glucosidase						
Gilbertella persicaria	17.5±0.9[b]	73.4±3.1[b]	15.4±0.7[c]	142.2±11.1[b]	13.8±1.1[b]	113.5±8.8[b]
Mucor corticolus	86.2±5.7[a]	83.2±4.6[a]	96.4±6.2[a]	89.9±4.9[c]	110.9±6.4[a]	87.3±5.9[c]
Rhizomucor miehei	4.5±0.4[d]	19.4±1.3[d]	5.7±0.6[d]	30.2±2.8[d]	6.1±0.6[c]	22.9±2.1[d]
Rhizopus niveus	12.0±0.9[c]	47.9±2.4[c]	20.4±1.5[b]	158.3±10.5[a]	13.1±0.7[b]	143.8±9.8[a]

*Values are mean±standard deviation of endoglucanase, cellobiohydrolase and β-glucosidase production by the four isolates after 2 days in SSF1 (F = 793.873, 203.639, 834.341) and SSF2 (F = 362.541, 193.333, 426.961), after 6 days in SSF1 (F = 1432.814, 609.937, 838.496) and SSF2 (F = 912.724, 63.830, 252.477) and after 10 days in SSF1 (F = 418.698, 333.757, 1165.546) and SSF2 (F = 243.120, 179.091, 248.937), respectively. Values within a column of each of the three enzymes followed by different lower case letters are significantly different according to ANOVA (Duncan's multiple range test) at df = 3.16 and p<0.05.

yield of this fungus had continuously increased exceeding 12.2 U g^{-1} dss activity to the tenth day of incubation. On the contrary, CBH production of the other strains displayed a decrease to the end of the fermentation. Anyway, the abovementioned *M. corticolus* CBH activity was the best value that could be measured in the two fermentation systems. In SSF2, *G. persicaria, R. miehei* and *Rh. niveus* showed their maximum CBH activity on the sixth day (8.5, 3.7 and 5.8 U g^{-1} dss) while *M. corticolus* reached it on the tenth day (8.2 U g^{-1} dss). Because maximal CBH concentrations were reached at different days of culturing by each strain, determination of relative catalytic activity is difficult to this enzyme.

β-Glucosidase activity

β-Glucosidase activity of the tested isolates was generally higher than the CMCase and CBH. Best activity was 158.3 U g^{-1} dss presented by *Rh. niveus* in SSF2. All strains exhibited its maximal enzyme activity on the sixth day of SSF2 where the relative β-glucosidase activity was *Rh. niveus > G. persicaria > M. corticolus > R. miehei*. A slight decrease in the enzyme activity could be observed after sixth day. In contrast to SSF2, β-glucosidase activity in SSF1 varied and maximum enzyme activities were measured after different incubation periods. For instance, *M. corticolus* and *R. miehei* demonstrated their best enzymatic activity on the tenth day (110.9 and 6.1 U g^{-1} dss), while *G. persicaria* and *Rh. niveus* exhibited their highest activity on the second (17.5 U g^{-1} dss) and the sixth day (20.4 U g^{-1} dss), respectively. It is
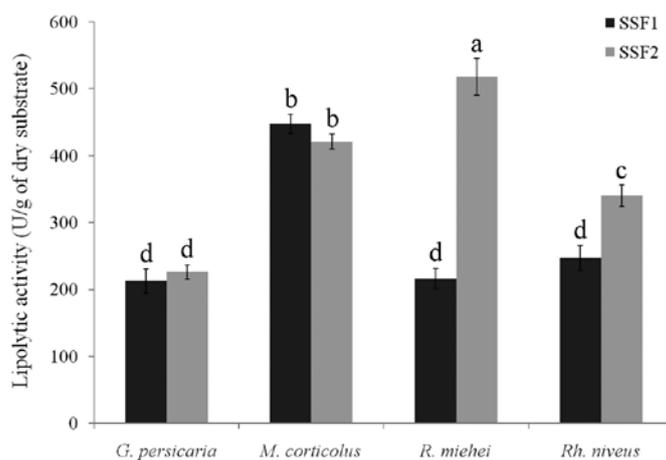

Fig. 2. Maximal lipolytic activity of zygomycetes strains in corn residue-based (SSF1) and mixed (corn residue and wheat bran) substrate-based (SSF2) solid-state fermentations. Presented activities were measured on the 2nd day of fermentation by *G. persicaria,* and 12th day by *M. corticolus, R. miehei* and *Rh. niveus.* Bars represent SD values of replicates. Different letters over the bars indicate significant differences (P<0.05) in Duncan's multiple range test

worth to mention that similar to its CBH production, β-glucosidase yield of *M. corticolus* in SSF1 was superior to that reached in SSF2. This enhanced activity maintained throughout the whole cultivation period.

Lipolytic enzyme production

Lipase yield of *G. persicaria* reached its maximum after two-day cultivation; *M. corticolus, R. miehei* and *Rh. niveus* had their maximum enzyme activity on the 12th incubation day. Cultivation of *R. miehei* and *Rh. oryzae* in SSF2 resulted considerably higher lipase yield than in SSF1. As shown in Figure 2, *R. miehei* had the highest *p*NPP hydrolyzing activity (518 U g^{-1} dss) after fermentation for 12 days. Almost 2.5 times higher lipase yield was detected at this fungus in SSF2 compared to that in SSF1. *M. corticolus* was the best lipase producer in fermentation system containing only corn residues as substrate (see SSF1 in Fig. 2). The presented 448 U g^{-1} dss enzyme yield was about 2 times higher than those measured in the crude extracts of the other strains. The relative lipase yield was *M. corticolus > Rh. niveus > R. miehei > G. persicaria* in SSF1, and *R. miehei > M. corticolus > Rh. niveus > G. persicaria* in SSF2.

Proteolytic enzyme production

Protease production of the *Rh. niveus* strain was outstanding among the tested isolates in both SSF1 and SSF2. However, only the assay of Suc-AAPF-*p*NA hydrolysis showed efficient results by this fungus, which refers to a strong chymotrypsin-like
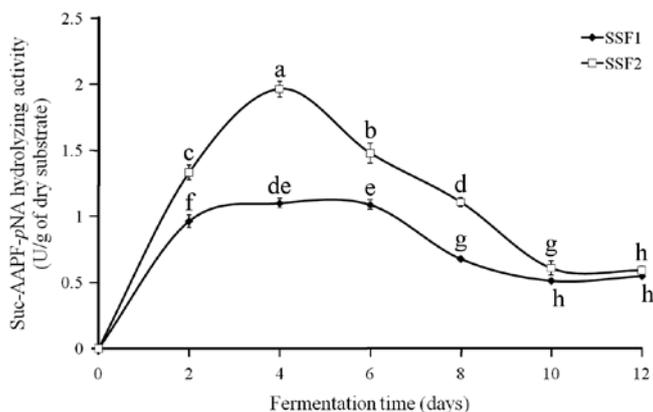

Fig. 3. Time course of Suc-AAPF-*p*NA degrading protease production by *Rh. niveus* under corn residue-based (SSF1) and mixed (corn residue and wheat bran) substrate-based (SSF2) solid-state fermentations. Bars represent SD values of replicates. Different letters on the bars are significantly different (P<0.05) in Duncan's multiple range test

proteolytic activity. The presented 1.96 U g^{-1} dss maximum proteolytic activity was generally 4 to 10 times higher than those of the other isolates. *G. persicaria* also had mentionable Suc-AAPF-*p*NA hydrolyzing activity on the fourth day in SSF2 (0.52 U g^{-1} dss). Chymotrypsin-like proteolytic activity of the other tested strains was remained in the range from 0.15 to 0.39 U g^{-1} dss. The Bz-R-*p*NA and Bz-FVR-*p*NA hydrolyzing activities generally varied between 0.024 and 0.37 and 0.014 and 0.43 U g^{-1} dss, respectively, which indicated moderate trypsin-like proteolytic activities of the isolates. *M. corticolus* showed the highest activity values for both trypsin substrates. Figure 3 depicts the time course profile of chymotrypsin-like protease activity of *Rh. niveus* during the 12-day incubation period. The enzyme production reached a maximum level after 96 h followed by a slight decrease up to the end of the fermentation. Similarly to the most of the other tested enzymes, SSF2 proved to be a better medium for chymotrypsin-like protease production.

DISCUSSION

Isolates selected for this study proved to be good extracellular β-glucosidase and/or lipase producers in our previous experiments [17, 31]. In that studies, SSF on wheat bran generally resulted in significantly higher enzyme activities than the liquid cultures. Hence, two SSF systems were tested in the present assays: a single medium of chopped corn stalks and leaves (SSF1) and a mixed substrate containing corn residues and wheat bran (SSF2).

Each isolate showed intensive growth under the applied culturing conditions, but they revealed noticeable differences in the production of the tested hydrolases. Generally, higher enzyme activities could be detected when SSF2 medium was used. It might be attributed to the nutritious properties of wheat bran. This plant residue supplies the fungi with convenient amount of nutrients, such as proteins, carbohydrates, fats, fiber, ash, minerals; furthermore, it has suitable particle size and porosity for oxygen supply [3]. Due to these properties, the crude fiber content of wheat bran might be highly accessible for the fungal exoenzymes. Our studies with different proportions of wheat bran also strengthened this suggestion (results not shown). In that assays, higher wheat bran concentration caused increased cellulase and lipase activities while proteolytic enzyme secretion did not change. In light of this viewpoint, it is important to note that the FPase activities on SSF1 were higher than those reported recently for other fungal cellulases on rice straw [22, 29] and oil palm trunk [1]. Kaur et al. [16] also used plant residue substrate in SSF to evaluate the cellulase production of mutants derived from heterokaryotic *Aspergillus* strains. Though CMCase, CBH and β-glucosidase activities of the 'A' (III) mutant developed in that study were 3.28, 5.32 and 7.03 times higher, respectively, than our best yields obtained for these enzymes (Table 1), FPase activities of *G. persicaria, R. miehei* and *Rh. niveus* on SSF2 were superior than this mutant strain. Cellulase preparations from these strains may be feasible additives for fast enzymatic pre-treatment of cellulosic wastes. This process is a critical step for bioethanol production, and requires enhanced

FPase activity in the crude cellulase extract [7]. Nevertheless, considering the FPase yields obtained on SSF1, the chopped corn stalk and leaf residues may also be useful as zygomycetous cellulase inducers. Moreover, *M. corticolus* presented higher CBH and β-glucosidase yields on SSF1 than SSF2, which also confirms the suitability of these plant wastes for cellulase production. In contrast, big differences were found in the cellulase activity of *R. miehei* on SSF1 and SSF2. Besides the slight CBH induction, the latter media provided about 4–5 times higher CMCase and β-glucosidase activities.

In both fermentation systems, CBH activity of the isolates was significantly lower than that of CMCase (Table 1). It is known that activities of these enzymes complement each other lead to synergy [32]; therefore, elevated CMCase activity generally causes enhancement in CBH activity. For that reason, it is worth mentioning that despite the high CMCase activity, moderate CBH production was detected in the case of *G. persicaria* as compared to the other strains. Additionally, production of CMCase usually reached its maximum during the exponential phase (second day), while CBH and β-glucosidase reached it during the late growth phase (sixth day or after). Elevated CMCase secretion has also been described at the beginning of the growth of *Aspergillus fumigatus* AKB9, when CMC and 2-deoxy-D-glucose were applied as substrates in submerged fermentation [7]. On the other hand, extracellular β-glucosidase activity of the tested fungi was found to be considerably higher than their CBH activity, which is able to hydrolyze microcrystal cellulose moiety. A similar observation was recorded for mixed cultures of *Aspergillus ellipticus* and *A. fumigatus* in SSF [14]. The 158.3 U g^{-1} dss β-glucosidase yield presented by *Rh. niveus* on the sixth day in SSF2 was about 1.74 times higher than those reported previously for this fungal strain (described as *Rh. stolonifer*) on wheat bran [31].

Wheat bran-based media have been reported as a good candidate for lipolytic enzyme induction in filamentous fungi [6, 10] including several zygomycetes [12, 17]. Since other plant-waste residues may also be able to increase the lipase yield, testing of them in both submerged and solid fermentations has continuous attention. The corn stalk and leaf materials examined in this study provided enhanced lipolytic activities (Fig. 2), which are comparable to lipase yields presented by other filamentous fungi in plant-waste residue-based fermentations [25, 34]. The lipolytic enzyme production of *M. corticolus* was slightly better on SSF1 than SSF2, which is similar to that observed for the cellulases. Among the tested fungi, *M. corticolus* seems to be the most promising fungus for cellulase and lipase secretion on corn stalk and leaf residues. Lipolytic enzyme production of *G. persicaria* on SSF2 was considerably lower than that of the other strains; unlike its remarkable cellulase yields [31]. This fungus had weak lipid hydrolyzing capacity in our previous tests, too [17].

It is well known that agricultural by-products can also be utilized for proteolytic enzyme production by filamentous fungi [35]. Different plant wastes were tested to induce the protease secretion of *Aspergillus flavus,* wherein wheat bran proved to be the best substrate [21]. Wheat bran is also preferred for protease production by zygomycetes and several *Mucor, Rhizopus* and *Rhizomucor* strains have been identified as promising sources of these enzymes [11]. Hydrolysis of peptidyl-*p*NAs showed

induced chymotrypsin-like protease secretion by *Rh. niveus* under both SSF conditions. The secreted proteolytic enzyme exhibited the highest activity towards the chymotrypsin substrate Suc-AAPF-*p*NA, indicating that it has endoproteolytic activity, and a preference for phenylalanine. Similar substrate specificities have been described for alkaline proteases from *A. fumigatus* [18] and *Fusarium culmorum* [23]. The enzyme from *F. culmorum* was considered to be a subtilisin-like protease, which implies that the extracellular proteolytic activity of *Rh. niveus* may relate to fungal subtilisins. The saprotroph nature of the isolate may also strengthen this suggestion since the endoprotease activities of such fungi are mainly provided by subtilisin-like enzymes [9].

In conclusion, the cellulase and lipase production of the tested Mucoromycotina fungi proved to be inducible when corn stalk and leaf plant-waste residues were used in SSF conditions. Nonetheless, as we know, this is the first report on the protease activity of *Rh. niveus* under SSF using corn residues. Further studies are needed in order to set up the appropriate fermentation parameters through statistical methodologies, and to isolate and characterize the identified *Rh. niveus* proteolytic enzyme.

ACKNOWLEDGEMENTS

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